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Transgenic Zebrafish Reveal Tissue-Specific Differences in Estrogen Signaling in Response to Environmental Water Samples

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Running title: Tissue-specific effects of endocrine disruptors

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Abstract

Background: Environmental endocrine disruptors (EED) are exogenous chemicals that mimic endogenous hormones, such as estrogens. Previous studies using a zebrafish transgenic reporter demonstrated that the EEDs bisphenol A and genistein preferentially activate estrogen receptors (ER) in the larval heart compared to the liver. However, it was not known whether the transgenic zebrafish reporter was sensitive enough to detect estrogens from environmental samples, whether environmental estrogens would exhibit similar tissue-specific effects as BPA and genistein or why some compounds preferentially target receptors in the heart.

Methods: We tested surface water samples using a transgenic zebrafish reporter with tandem estrogen response elements driving green fluorescent protein expression (5xERE:GFP). Reporter activation was colocalized with tissue-specific expression of estrogen receptor genes by RNA *in situ* hybridization.

Results: Selective patterns of ER activation were observed in transgenic fish exposed to river water samples from the Mid-Atlantic United States, with several samples preferentially activating receptors in embryonic and larval heart valves. We discovered that tissue-specificity in ER activation is due to differences in the expression of estrogen receptor subtypes. ERα is expressed in developing heart valves but not in the liver, whereas ERβ2 has the opposite profile. Accordingly, subtype-specific ER agonists activate the reporter in either the heart valves or the liver.

Conclusion: The use of 5xERE:GFP transgenic zebrafish has revealed an unexpected tissue-specific difference in the response to environmentally relevant estrogenic compounds. Exposure to estrogenic EEDs *in utero* is associated with adverse health effects, with the potentially unanticipated consequence of targeting developing heart valves.

Introduction

Estrogens are small molecules that influence organ formation and function (Deroo and Korach 2006). Estrogens bind to and activate receptors in the cytosol, which then travel to the nucleus and directly regulate gene expression. Multiple estrogen receptor (ER) genes are present in vertebrates, such as the *Esr1* and *Esr2* genes in mice (coding for ERα and ERβ proteins) and the *esr1*, *esr2a* and *esr2b* genes in zebrafish (coding for ERα, ERβ1 and ERβ2 proteins). Exposure to environmental endocrine disruptors (EED) that bind to ERs are associated with increased risk of cancers and abnormal reproductive tract formation in mammals and fish (Ma 2009). Because ERs are expressed widely in many tissues (Kuiper et al. 1997), exposure to estrogenic EEDs may also influence the development of non-reproductive tissues (Meeker 2012). Therefore, detecting environmental estrogens and identifying their sites and mechanism of action during organismal development is of paramount importance.

Standard methods to detect ER activity use yeast and mammalian cell culture assays (Legler et al. 1999; Leskinen et al. 2005; Routledge and Sumpter 1996; Sanseverino et al. 2005) that are limited in their utility because they are not representative of tissue diversity. Additionally, while these methods can demonstrate the presence of estrogenic chemicals in environmental samples, they do not address whether chemicals are being absorbed and producing an effect at the organismal level. ER activity assays have been developed for fish and mice, however most reporter constructs are designed to act in certain tissues exclusively (such as liver or brain) (Brion et al. 2012; Kurauchi et al. 2005) or have used a bioluminescent reporter (such as luciferase) that has limited spatial resolution (Ciana et al. 2003; Legler et al. 2000).

Previously, we developed transgenic zebrafish that specifically report estrogen receptor transcriptional activity in all tissues of embryos and larvae with single cell resolution (Gorelick and Halpern 2011). The reporter line contains tandem estrogen response element DNA

sequences (Gruber et al. 2004) driving green fluorescent protein (GFP) expression (5xERE:GFP). The 5xERE:GFP line serves as a tissue-specific reporter of estrogen receptor-mediated transcriptional activity following exposure of zebrafish embryos to estrogenic compounds. Exposure to certain purified compounds results in preferential activation of GFP in heart valves, whereas other compounds activate the reporter only in the liver (Gorelick and Halpern 2011). Similar results were reported independently using a 3xERE zebrafish reporter (Lee et al. 2012). We sought to determine whether the ER reporter zebrafish would also be useful to detect the presence of environmental estrogens and to discover the basis for the tissue-specific differences in response to estrogens.

Methods

Materials and zebrafish husbandry

Estradiol (purity ≥ 98%), bisphenol A (BPA, purity ≥ 99%) and dimethyl sulfoxide (DMSO, purity ≥ 99.9%) were purchased from Sigma-Aldrich (MO, USA). 7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780), 2,3-***bis***(4-Hydroxyphenyl)-propionitrile (DPN), 4,4',4''-(4-Propyl-[1***H***]-pyrazole-1,3,5-triyl)***tris***phenol (PPT), 1,3-***Bis***(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1***H***-pyrazole dihydrochloride (MPP) and 4-[2-Phenyl-5,7-***bis***(trifluoromethyl)pyrazolo[1,5-***a***]pyrimidin-3-yl]phenol (PHTPP) were obtained from Tocris Biosciences (Bristol, UK), with purity > 99% except for MPP, with purity > 98%. All chemicals were dissolved in DMSO and diluted into dechlorinated fish water (water used to house zebrafish, UV sterilized and circulated through a fluidized bed filtration system, Aquaneering, Inc.) such that the final concentration of DMSO was 0.1%. Zebrafish strains used were the wild type AB laboratory strain (Walker 1999) and *Tg(5xERE:GFP)*$^{c262/c262}$ and *Tg(5xERE:GFP)c263* (Gorelick and Halpern 2011). All work was

approved by the Carnegie Institution Institutional Animal Care and Use Committee. All animals were treated humanely and with regard for alleviation of suffering.

RNA *in situ* hybridization

Antisense RNA probes corresponding to *esr1* (ERα), *esr2a* (ERβ1) and *esr2b* (ERβ2) were used as described (Gorelick and Halpern 2011) (note that some previous publications (Bertrand et al. 2007) refer to *esr2b* gene as *esr2a* and *esr2a* as *esr2b*). Sense RNA probes were also assayed but did not produce signals above background (data not shown). Colorimetric whole-mount in situ hybridization on zebrafish embryos and larvae was performed as described (Gorelick and Halpern 2011) with the modification that 5% dextran (final) was included in the hybridization solution (Lauter et al. 2011). Images were collected using a Zeiss Axioskop microscope equipped with an AxioCam HRc digital camera (Carl Zeiss Microimaging, Thornwood, NJ). Image adjustments and cropping were performed using Photoshop CS5 and InDesign CS5 (Adobe Systems Inc., San Jose, CA).

Water sampling

To concentrate estrogens over time, passive sampling devices (Polar Organic Chemical Integrative Sampler (POCIS) fabricated at the Columbia Environmental Research Center as described (Alvarez et al 2004)) were deployed in rivers and streams at 19 locations in the Shenandoah watershed and Allegheny, Delaware and Susquehanna Rivers in Virginia and Pennsylvania in April 2010 for 31-45 days (see Supplemental Material, Table S1). The Shenandoah and Susquehanna sites are part of an ongoing monitoring and research program to determine the factors involved in fish lesions and mortalities and to assess signs of reproductive endocrine disruption (testicular oocytes and plasma vitellogenin in male bass) observed in theses watersheds (Blazer et al. 2010; Reif et al. 2012). The Allegheny and Delaware sites were used as comparisons for the Susquehanna sites in the Pennsylvania emerging contaminants project (Reif

et al. 2012). POCIS devices were also deployed during April and May as these times were previously identified as periods of high estrogenicity in the VA watershed (Ciparis et al. 2012). After 31-45 days, the sampling devices were retrieved as described (Alvarez 2010) and POCIS membranes were shipped to the United States Geological Survey, Columbia Environmental Research Center (http://www.cerc.usgs.gov/Branches.aspx?BranchId=14) for analyte recovery as previously described (Alvarez et al. 2009). Briefly, the POCIS membranes were extracted using 50 mL of 1:1:8 (V:V:V) methanol:toluene:dichloromethane followed by 20 mL of ethyl acetate. Extracts were reduced by rotary evaporation, filtered, and composited into 2-POCIS equivalent samples, thereby increasing the amount of chemical present in each sample to aid in detection. Samples were resuspended in DMSO and diluted into fish water between 100 and 4000 fold. At 1-day post fertilization (d) *Tg(5xERE:GFP)*$^{c262/c262}$ embryos were exposed to treated water and at 3 or 4 d examined for fluorescent labeling. Four embryos were exposed per treatment (see Supplemental Material, Table S2). Embryos were incubated in 24- or 96- well plates at a density of no more than 4 or 2 embryos per well, respectively. Exposure occurred under static water conditions with no water changes during exposure. Embryos were incubated at 28°C under an 18 hour light 6 hour dark cycle.

For discrete water sampling, two sites from the POCIS deployment were selected for follow-up analysis based on results from the initial zebrafish assay. Muddy Creek was selected because samples from this site preferentially activated the reporter in heart valves. Hawksbill Creek was selected because samples from this site exhibited the most intense fluorescence. Water was collected from the Muddy Creek and Hawksbill Creek locations (corresponding to samples 7 and 16 from the POCIS study, see Supplemental Material, Table S1) approximately one year after passive sampling to minimize seasonal effects. Samples were extracted with OASIS HLB as described (Ciparis et al. 2012). The methanol/methanol:DCM eluate was dried under a continuous flow of atmospheric air, resuspended in DMSO and serially diluted into fish water

between 500 and 10,000 fold (equivalent to exposing larvae to between 5 and 100 fold concentrated water).

For negative controls, a field blank was prepared for a POCIS site and treated identically to POCIS extractions as described (Alvarez 2010). Briefly, field blanks are stored in airtight containers and transported to the field locations in insulated coolers. During both deployment and retrieval of the passive samplers the lids of the field blank containers are opened and exposed to the surrounding air. This simulates possible exposure to air-borne contaminants of the actual deployed sampler. The field blanks are then extracted identically to the deployed sampler. For a vehicle control, zebrafish were incubated in fish water containing 0.1% DMSO, except for conditions when POCIS samples were diluted 1:100 into fish water, in which case vehicle control was 1% DMSO. For positive controls, zebrafish were incubated in water containing 100 ng/ml estradiol. Samples were randomly coded so that the experimenter was blind to their identity during testing on zebrafish. For the initial screening, GFP fluorescence within live embryos and larvae was visualized using an Olympus MVX10 fluorescent stereomicroscope equipped with a Leica DCF500 digital camera. Images were captured using identical microscope and camera settings. For secondary imaging at higher magnification, embryos and larvae were mounted on bridged coverslips and examined on a Zeiss Axio Imager microscope equipped with an AxioCam HRm digital camera.

Morpholinos

To reduce levels of ER protein, 1 cell-stage *Tg(5xERE:GFP)*$^{c262/c262}$ embryos were injected with antisense morpholino oligonucleotides targeting the translation start sites of *esr2a* (5'-ACATGGTGAAGGCGGATGAGTTCAG) or *esr2b* (5'-AGCTCATGCTGGAGAACACAAGAGA) (Gene Tools, Philomath OR). Morpholinos were resuspended in water at 30 μM and 1-2 nl injected into each embryo as described (Nasevicius

and Ekker 2000). Beginning at 2 days post fertilization (d), embryos were incubated in 10 μM BPA or vehicle control (0.1% ethanol). At 3 d, fluorescence was assayed as described above.

Yeast estrogen receptor reporter assay

To measure estrogen equivalents (relative to 17β-estradiol) of the analytes present in the POCIS extracts, a bioluminescent yeast estrogen screen (Sanseverino et al. 2005) using strain BLYES was performed as described (Ciparis et al. 2012). All assay plates included a 12-point standard curve consisting of estradiol (2.3×10^{-11} - 5.0×10^{-7} M) and sample blanks containing minimal media only. Samples, standards and blanks were run in triplicate. Luminescence was quantified using a SpectraFluor Plus plate reader (Tecan Group Ltd, Durham, NC). A linear calibration curve was created using $\log_{10}$ transformations of the five lowest standards (2.3×10^{-11} – 2.1×10^{-10} M estradiol) and their associated mean luminescence. Concentrations in samples with luminescence above this range were quantified using four points from the linear portion of the dose-response curve ($\log_{10}$[estradiol] vs. mean luminescence; 1.2×10^{-10} - 1.9×10^{-9} M estradiol), extrapolated from these standards and reported as ng/POCIS estradiol equivalents (E2Eq).

Results

Environmental estrogens preferentially activate receptors in heart valves

Groups of 1 day post fertilization (d) 5xERE:GFP transgenic zebrafish embryos were exposed for 3-4 days to POCIS extracts collected from 19 locations in the Shenandoah River watershed and the Allegheny, Delaware and Susquehanna Rivers (Figure 1 and Supplemental Material, Table S1). A surprisingly large number of samples (16) activated the ER reporter in transgenic zebrafish, with 3 samples preferentially inducing GFP labeling of the heart valves (Figure 1 and Supplemental Material, Table S2 and Movie S1). Exposure to sample numbers 3 (Delaware River, diluted 1:1000) and 6 (Naked Creek, Virginia, diluted 1:500) caused activation of the ER

reporter in the heart valves but not the liver (Supplemental Material, Table S2). Embryos exposed to sample 7 (Muddy Creek, Virginia) showed activation in both tissues, but with increased sensitivity in the heart valves (1:1000 dilution) compared to the liver (1:500 dilution).

To confirm that reporter activity was specific for estrogen receptors, we exposed embryos to water samples that either activated the reporter in the heart valves alone (samples 3 or 7 diluted 1:1000) or together with the liver (samples 16 or 18 diluted 1:1000) in the presence of the ER antagonist ICI 182,780 (Robertson 2001). Co-treatment with 10 μM ICI 182,780 abolished fluorescence in all embryos (Figure 2B, D and Supplemental Material, Table S2), indicating that the chemicals in the water are either ER agonists or lead to the production of ER agonists in zebrafish. Embryos treated with 100 ng/ml estradiol exhibited robust fluorescence in the heart and liver (Figure 2G), while embryos treated with 10 μM ICI 182,780 alone did not exhibit fluorescence (data not shown), consistent with previous studies (Gorelick and Halpern, 2011). Thus, 5xERE:GFP transgenic zebrafish larvae can report tissue-specific ER activation of unknown estrogens from passively sampled water.

POCIS sampling provides a time-weighted average of chemical exposure over several weeks, whereas discrete sampling provides a snapshot of chemical exposure at a single point in time. We examined whether the zebrafish reporter was sensitive enough to detect environmental estrogens from single pass collections at the Muddy Creek and Hawksbill Creek locations (samples 7 and 16 from the passive sampling study, Supplemental Material, Table S1). Approximately one year following passive sampling, we collected and concentrated 1L of water from the same locations. As in the previous findings, water from Hawksbill Creek diluted 1:500 or 1:1000 activated the reporter in the heart valves, whereas at greater dilutions (1:5000, 1:10,000) fluorescence was not observed (Figure 2, compare C and F, n = 20 embryos per

dilution). Thus, 5xERE:GFP embryos can detect environmental estrogens from water samples collected from the same sites at different times using passive or discrete sampling methods.

To assess the sensitivity of the 5xERE:GFP zebrafish reporter, we compared the responses in zebrafish to those measured with a widely used yeast reporter assay (Sanseverino et al. 2005; Leskinen et al. 2005; Balsiger et al. 2010). Passively sampled water was tested using the bioluminescent yeast estrogen screen (BLYES; Sanseverino et al. 2005), which utilizes a yeast strain containing the human ERα gene and a tandem ERE that drives an inducible *luxAB* reporter. Every water sample that activated the zebrafish reporter was readily detected using the yeast system (estradiol equivalents between 0.8 and 8 ng/POCIS; Supplemental Material, Table S2). Moreover, the three water samples that failed to activate the zebrafish reporter exhibited the lowest levels of activity in the yeast assay (< 0.8 estradiol equivalents, Supplemental Material, Table S2).

Tissue-specificity in estrogen receptor gene expression

A plausible explanation for the tissue-specific differences in activation of the transgenic reporter by known estrogenic compounds and environmental samples is diversity in estrogen receptors. Zebrafish express three ER subtypes, ERα, ERβ1 and ERβ2 (encoded by the *esr1*, *esr2a* and *esr2b* genes), which, similar to their mammalian orthologues, bind ligands with different affinities in vitro and in cultured cells (Cosnefroy et al. 2012; Menuet et al. 2002). Previous studies demonstrated that *esr2b* is expressed in the embryonic and larval liver (Bertrand et al. 2007; Gorelick and Halpern 2011). However, there has been no report of ER transcripts or proteins in the developing heart of zebrafish.

For this reason, we re-examined ER gene expression in 3-5 d zebrafish using a method for whole-mount in situ hybridization with enhanced sensitivity (Lauter et al. 2011). In 5 d zebrafish larvae, we observed robust expression of *esr2b* in the liver, consistent with previous studies

(Bertrand et al. 2007; Gorelick and Halpern 2011), and discovered that *esr1* is selectively transcribed in the developing valves of the heart (Figure 3). *esr2a* transcripts were not detected at these stages (Figure 3B). The results demonstrate that different ER subtypes are specifically expressed in the heart and liver. Previous studies demonstrated that the ER ligands bisphenol A and genistein preferentially activate receptors in zebrafish heart valves compared to the liver (Gorelick and Halpern, 2011). The differences in ER subtype localization reported here support the idea that BPA and genistein preferentially activate ERα in the heart because they have a higher affinity for this ER subtype.

Selective estrogen receptor modulation in the heart and liver

To corroborate the findings of differential gene expression, we used genetic and pharmacological approaches to activate or inhibit ERα, ERβ1 or ERβ2 selectively in 3-4 d transgenic zebrafish. On the basis of gene expression, reducing ERβ2 protein levels should reduce estrogen receptor activity in the liver but not in heart valves. We injected 5xERE:GFP embryos with antisense morpholino oligonucleotides targeting *esr2a* or *esr2b* genes (1-2 nl of 30 μM solution), incubated embryos in 10 μM BPA and assayed fluorescence. Fluorescence in the liver was reduced in *esr2b*-morphant embryos compared to *esr2a*-morphant embryos, whereas robust labeling of the heart valves was observed in all morphant embryos (Figure 4A, B; Table 1). Embryos exposed to vehicle control exhibited no fluorescence in the liver or heart (not shown). Attempts to reduce ERα levels using three different morpholinos targeting *esr1* gene transcription and RNA splicing were ineffective as *esr1*-morphant embryos exhibited pleiotropic developmental defects such as cardiac edema, a small head and curved tail (not shown), suggesting a non-specific response.

To activate ER subtypes selectively we used the synthetic ER ligands propylpyrazoletriol (PPT) and diarylpropionitrile (DPN) (Meyers et al. 2001; Stauffer et al. 2000). PPT has higher affinity

for human ERα than for ERβ, while DPN has higher affinity for ERβ. We found, however, that 5xERE:GFP zebrafish embryos exposed to 100 μM PPT showed GFP labeling of only the liver where ERβ2 is produced (n=10), while those exposed to 1 μM DPN showed GFP-labeling of the heart valves that synthesize ERα (n=20, Figure 4D, E). As a positive control, embryos exposed to 10 μM BPA showed GPF-labeling of the heart valves and liver (Figure 4C). To inhibit ER subtypes selectively we exposed zebrafish to selective antagonists designed against human ER subtypes. Treatment of 5xERE:GFP embryos with either the ERα or ERβ antagonists methyl-piperidino-pyrazole (MPP) (Sun et al. 2002) or 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl)phenol (PHTPP) (Compton et al. 2004) failed to inhibit reporter activity in any tissue (data not shown). Thus, different ER agonists selectively activate receptors in either the heart valves or the liver of zebrafish larvae, but in an opposite manner to what was expected based on their activation of the human ER receptors.

Discussion

Here we demonstrate that 5xERE:GFP reporter zebrafish can detect the tissue specific effects of environmental estrogens. This represents a significant improvement over traditional detection assays using yeast (Routledge and Sumpter 1996) or cultured cells (Legler et al. 1999), which do not allow comparisons between multiple tissues. Furthermore, testing compounds for ER activity in zebrafish larvae involves the physiologically relevant parameters of absorption, distribution, metabolism and excretion.

We found a high concordance between responses in zebrafish and in a bioluminescent yeast assay for detection of estrogens from the same environmental samples. This indicates that the whole embryo assay of transgenic zebrafish correlates well with an established and sensitive method (Ciparis et al. 2012; Di Dea Bergamasco et al. 2011; Sanseverino et al. 2005) for measuring estrogenic compounds in water samples. Additionally, the zebrafish reporter revealed

a previously unknown tissue and developmental stage for ER signaling, the newly formed heart valves. With the genetic and pharmacological tools available to manipulate zebrafish, transgenic models can be readily applied to detect tissue-specific environmental estrogens and identify their mode of action. Future studies will broaden this approach to report the activity of other environmentally relevant small molecules such as androgens and dioxins.

Although 5xERE:GFP zebrafish were able to detect estrogens in discrete and time-integrated passively collected water samples, our results suggest that estrogen levels vary depending on the sampling method. For example, POCIS extracts prepared from water collected from Muddy Creek in June 2010 activated the reporter preferentially in heart valves, whereas discrete water samples collected the following year did not. Similarly, POCIS extracts from Hawksbill Creek collected in June 2010 activated the reporter in the heart valves and liver, but discrete water samples collected the following year preferentially activated the reporter in heart valves. These differences are not surprising, however, given the likely daily and seasonal variations in the concentration of environmental estrogens (Ciparis et al. 2012; Martinovic et al. 2008).

An unexpected finding is that that DPN and PPT appear to activate zebrafish ERα and ERβ2, respectively, the opposite of what has been observed for the human ER subtypes (Meyers et al. 2001; Stauffer et al. 2000). One possibility is that zebrafish ERα has greater functional homology to human ERβ. Although zebrafish ERα is most similar to human ERα when comparing the entire protein sequence, similarities between functional domains within each protein are more relevant for predicting functional homology. For example, in the N-terminal AF-1 domain that regulates transcriptional activation (also referred to as the A/B domain) (Metzger et al. 1995), zebrafish ERα is more similar to human ERβ (13.2%) than to human ERα (8.4%) (Menuet et al. 2002). Low sequence homology (< 15% identity) between the AF-1 domains from human and zebrafish ERs makes it difficult to predict functional homology between subtypes with accuracy.

Furthermore, studies using chimeric ER proteins from rainbow trout and humans suggest that, despite low sequence homology, estrogen receptor domains from different species may function similarly and interact with the same transcription factors (Petit et al. 2000). It is therefore not surprising that agonists might show altered affinities for ERs in species as diverse as fish and human.

Although ER-subtype selective agonists (DPN and PPT) designed against human ERs were effective in zebrafish, selective antagonists designed against human ER subtypes were not. These in vivo results are consistent with those obtained in cultured cells expressing zebrafish ERs, where MPP and PHTPP also failed to inhibit ERE-dependent reporter activity induced by 17α-ethynylestradiol (Notch and Mayer 2011). Together, this data suggests that MPP and PHTPP do inhibit zebrafish ERs.

The environmental estrogenic compound(s) that is capable of activating the zebrafish reporter with tissue-specificity remain to be identified. The low levels of known estrogens in water samples make this a challenging endeavor, requiring sequential rounds of HPLC fractionation for purification and mass spectrometry for identification. However, the small size and transparency of zebrafish embryos are advantageous for rapid, high-throughput screening of fractions for tissue-specific ER activity. Ultimately, it will be possible to identify unknown EEDs that affect estrogen signaling, their sites of action and effects on embryonic development.

The activation of estrogen receptors in heart valves during development leads to the intriguing hypothesis that estrogen signaling influences valve formation. In humans, the occurrence of heart valve abnormalities differs between the sexes, which could be due to sex differences in estrogen levels. Bicuspid aortic valve defects, where the aortic valve develops two leaflets instead of three, are four times more prevalent in men than in women (Warnes 2008). Since ERs are ligand-dependent transcription factors, it will be important to identify which genes are directly regulated

by estrogens and test whether they are important for cell migration or proliferation of valve precursors. Exposure to environmental endocrine disrupting compounds that mimic or inhibit endogenous estrogens *in utero* is associated with adverse health effects (Soto and Sonnenschein 2010), with the potentially unanticipated consequence of causing heart valve malformations.

References

Alvarez, DA. 2010. Guidelines for the use of the semipermeable membrane device (SPMD) and the polar organic chemical integrative sampler (POCIS) in environmental monitoring studies. U.S. Geological Survey, Techniques and Methods 1–D4, 28 p.

Alvarez DA, Cranor WL, Perkins SD, Schroeder VL, Iwanowicz LR, Clark RC, et al. 2009. Reproductive health of bass in the Potomac, USA, drainage: Part 2. Seasonal occurrence of persistent and emerging organic contaminants. Environ Toxicol Chem 28(5):1084-1095.

Alvarez DA, Petty JD, Huckins JN, Jones-Lepp TL, Getting DT, Goddard JP, Manahan SE. 2004. Development of a passive, in situ, integrative sampler for hydrophilic organic contaminants in aquatic environments. Environ Toxicol Chem 23:1640–1648.

Balsiger HA, de la Torre R, Lee WY, Cox MB. 2010. A four-hour yeast bioassay for the direct measure of estrogenic activity in wastewater without sample extraction, concentration, or sterilization. Sci Total Environ 408(6):1422-1429.

Bertrand S, Thisse B, Tavares R, Sachs L, Chaumot A, Bardet P-L, et al. 2007. Unexpected novel relational links uncovered by extensive developmental profiling of nuclear receptor expression. PLoS Genetics 3(11):e188.

Blazer VS, Iwanowicz LR, Starliper CE, Iwanowicz DD, Barbash P, Hedrick JD, et al. 2010. Mortality of centrarchid fishes in the Potomac drainage: Survey results and overview of potential contributing factors. J Aquat Anim Health 22:190-218.

Brion F, Le Page Y, Piccini B, Cardoso O, Tong S-K, Chung B-c, et al. 2012. Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (*cyp19a1b*-GFP) zebrafish embryos. PLoS ONE 7(5):e36069.

Ciana P, Raviscioni M, Mussi P, Vegeto E, Que I, Parker MG, et al. 2003. In vivo imaging of transcriptionally active estrogen receptors. Nat Med 9(1):82-86.

Ciparis S, Iwanowicz LR, Voshell JR. 2012. Effects of watershed densities of animal feeding operations on nutrient concentrations and estrogenic activity in agricultural streams. Sci Total Environ 414:268-276.

Compton DR, Sheng S, Carlson KE, Rebacz NA, Lee IY, Katzenellenbogen BS, et al. 2004. Pyrazolo[1,5-a]pyrimidines: estrogen receptor ligands possessing estrogen receptor beta antagonist activity. J Med Chem 47(24):5872-5893.

Cosnefroy A, Brion F, Maillot-Marechal E, Porcher JM, Pakdel F, Balaguer P, et al. 2012. Selective activation of zebrafish estrogen receptor subtypes by chemicals by using stable reporter gene assay developed in a zebrafish liver cell line. Toxicol Sci 125(2):439-449.

Deroo BJ, Korach KS. 2006. Estrogen receptors and human disease. J Clin Invest 116(3):561-570.

Di Dea Bergamasco AM, Eldridge M, Sanseverino J, Sodre FF, Montagner CC, Pescara IC, et al. 2011. Bioluminescent yeast estrogen assay (BLYES) as a sensitive tool to monitor surface and drinking water for estrogenicity. J Environ Monit 13(11):3288-3293.

Gorelick DA, Halpern ME. 2011. Visualization of Estrogen Receptor Transcriptional Activation in Zebrafish. Endocrinology 152(7):2690-2703.

Gruber CJ, Gruber DM, Gruber IM, Wieser F, Huber JC. 2004. Anatomy of the estrogen response element. Trends Endocrinol Metab 15(2):73-78.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, et al. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors {alpha} and {beta}. Endocrinology 138(3):863-870.

Kurauchi K, Nakaguchi Y, Tsutsumi M, Hori H, Kurihara R, Hashimoto S, et al. 2005. In vivo visual reporter system for detection of estrogen-like substances by transgenic medaka. Environ Sci Technol 39(8):2762-2768.

Lauter G, Soll I, Hauptmann G. 2011. Multicolor fluorescent in situ hybridization to define abutting and overlapping gene expression in the embryonic zebrafish brain. Neural Dev 6:10.

Lee O, Takesono A, Tada M, Tyler CR, Kudoh T. 2012. Biosensor zebrafish provide new insights into potential health effects of environmental estrogens. Environ Health Perspect 120(7):990-996.

Legler J, van den Brink CE, Brouwer A, Murk AJ, van der Saag PT, Vethaak AD, et al. 1999. Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicol Sci 48(1):55-66.

Legler J, Broekhof JLM, Brouwer A, Lanser PH, Murk AJ, van der Saag PT, et al. 2000. A novel in vivo bioassay for (xeno-)estrogens using transgenic zebrafish. Environ Sci Technol 34(20):4439-4444.

Leskinen P, Michelini E, Picard D, Karp M, Virta M. 2005. Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. Chemosphere 61(2):259-266.

Ma L. 2009. Endocrine disruptors in female reproductive tract development and carcinogenesis. Trends Endocrinol Metab 20(7):357-363.

Martinovic D, Denny JS, Schmieder PK, Ankley GT, Sorensen PW. 2008. Temporal variation in the estrogenicity of a sewage treatment plant effluent and its biological significance. Environ Sci Technol 42(9):3421-3427.

Meeker JD. 2012. Exposure to environmental endocrine disruptors and child development. Arch Pediatr Adolesc Med 166(10):952-958.

Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O, et al. 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol Reprod 66(6):1881-1892.

Metzger D, Ali S, Bornert JM, Chambon P. 1995. Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. J Biol Chem 270(16):9535-9542.

Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA. 2001. Estrogen receptor-β potency-selective ligands: Structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J Med Chem 44(24):4230-4251.

Nasevicius A, Ekker SC. 2000. Effective targeted gene 'knockdown' in zebrafish. Nat Gen 26(2):216-220.

Notch EG, Mayer GD. 2011. Efficacy of pharmacological estrogen receptor antagonists in blocking activation of zebrafish estrogen receptors. Gen Comp Endocrinol 173(1):183-189.

Petit FG, Valotaire Y, Pakdel F. 2000. The analysis of chimeric human/rainbow trout estrogen receptors reveals amino acid residues outside of P- and D-boxes important for the transactivation function. Nucleic Acids Res 28(14):2634-2642.

Reif AG, Crawford JK, Loper CA, Proctor A, Manning R, Titler R. 2012. Occurrence of pharmaceuticals, hormones, and organic wastewater compounds in Pennsylvania waters, 2006-09. U.S. Geological Survey Scientific Investigations Report 2012-5106, 99 p.

Robertson JF. 2001. ICI 182,780 (Fulvestrant)--the first oestrogen receptor down-regulator--current clinical data. Br J Cancer 85 Suppl 2:11-14.

Routledge EJ, Sumpter JP. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15(3):241-248.

Sanseverino J, Gupta RK, Layton AC, Patterson SS, Ripp SA, Saidak L, et al. 2005. Use of Saccharomyces cerevisiae BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. Appl Environ Microbiol 71(8):4455-4460.

Soto AM, Sonnenschein C. 2010. Environmental causes of cancer: endocrine disruptors as carcinogens. Nat Rev Endocrinol 6(7):363-370.

Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, et al. 2000. Pyrazole ligands: Structure-affinity/activity relationships and estrogen receptor-α-selective agonists. J Med Chem 43(26):4934-4947.

Sun J, Huang YR, Harrington WR, Sheng S, Katzenellenbogen JA, Katzenellenbogen BS. 2002. Antagonists selective for estrogen receptor alpha. Endocrinology 143(3):941-947.

Walker C. 1999. Haploid screens and gamma-ray mutagenesis. Methods Cell Biol 60:43-70.

Warnes CA. 2008. Sex differences in congenital heart disease: Should a woman be more like a man? Circulation 118(1):3-5.

Table 1. *esr* morpholino results.

Morpholino target	Dose	GFP+ heart valves only	GFP+ liver only	GFP+ valves and liver	n embryos
esr2a	2 nl of 30 μM	0%	0%	100%	18
esr2b	1 nl of 30 μM	56%	0%	44%	18
esr2b	2 nl of 30 μM	94%	0%	6%	18

1-cell stage 5xERE:GFP embryos were injected with translation blocking morpholinos to reduce ER levels. At 2 days post fertilization (d), embryos were incubated in 10 μ M BPA. Fluorescence was assayed at 3 d as percent GFP-positive embryos (GFP+) in the indicated tissues.

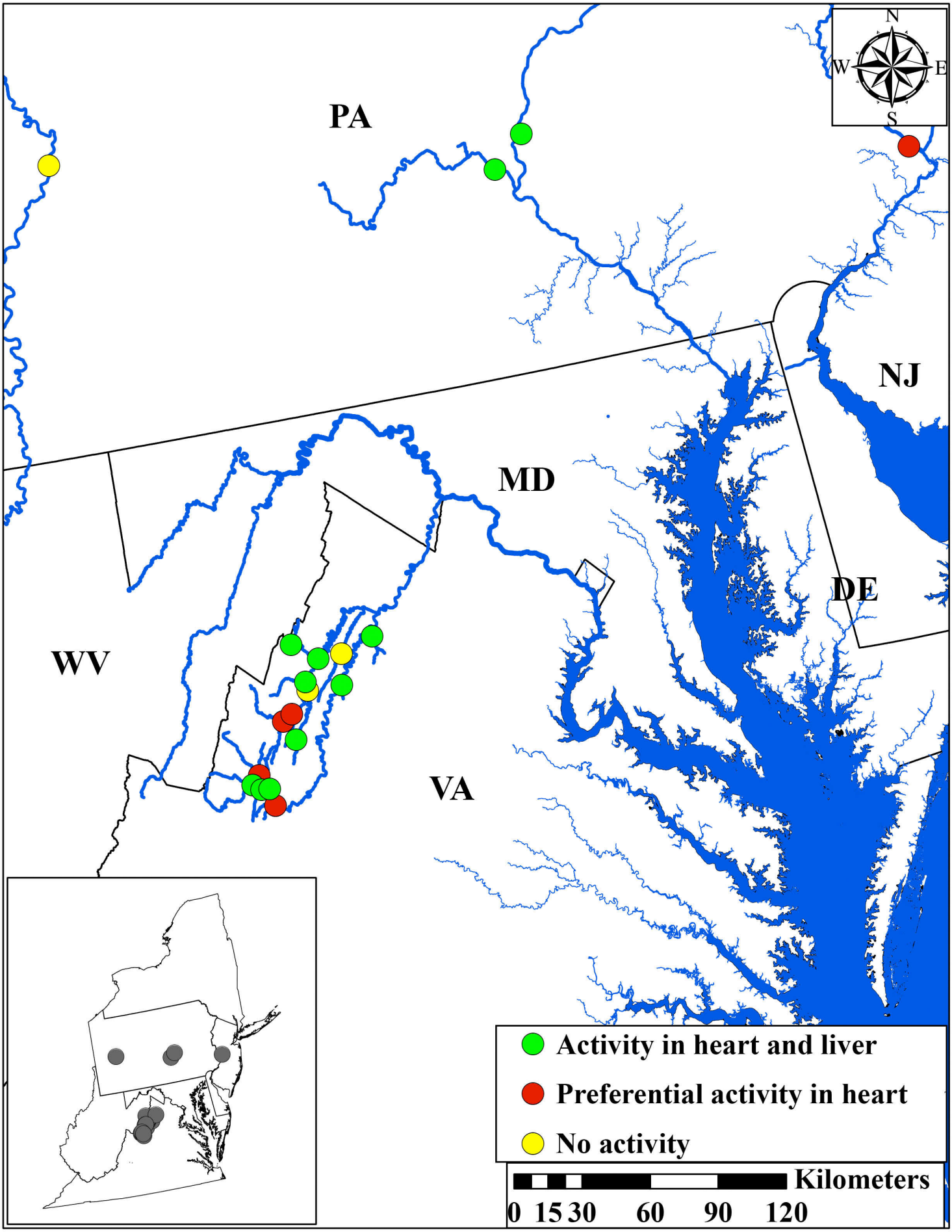
Figure Legends

Figure 1. Sites of sample collection. Circles denote POCIS deployment sites in April and May of 2010. Red denotes samples that preferentially activated estrogen receptors in heart valves, green denotes samples that activated estrogen receptors in heart valves and in liver and yellow denotes samples that did not activate receptors in the 5xERE:GFP zebrafish reporter. DE, Delaware; MD, Maryland; NJ, New Jersey; PA, Pennsylvania; VA, Virginia; WV, West Virginia.

Figure 2. Tissue-specific responses to environmental estrogens. A-F: *Tg(5xERE:GFP)*^{c262} zebrafish embryos were incubated in water containing extracts from water sampled from the Shenandoah Watershed and nearby rivers. Fluorescence was visualized in the liver (arrows) and heart valves (arrow heads) of 3-4 days post fertilization (d) live larvae. Water was passively sampled from (A) Delaware River, Pennsylvania (sample 3, diluted 1:1000), (B) Delaware River sample in the presence of estrogen receptor antagonist ICI 182,780 (10 μM), (C) Hawksbill Creek, Virginia (sample 16, diluted 1:1000), (D) Hawksbill with 10 μM ICI 182,780. (E) Naked Creek, Virginia (sample 6, diluted 1:500). (F) Water was discretely sampled from Hawksbill Creek the following year (diluted 1:500). (G) Larva incubated in positive control water containing 100 ng/ml estradiol. (H) Larva incubated in negative control field blank. Larvae are 4 d except for F, 3 d. All are lateral views, anterior to the left, dorsal to the top. Scale bar = 100 μm.

Figure 3. Specific expression of the *esr1* gene in heart valves. Estrogen receptor gene expression was detected using whole mount in situ hybridization on 5 day old larvae. A: *esr1* transcripts are in heart valves (arrow heads) but not in liver. Inset, high magnification ventral view of heart showing labeling of atrioventricular valve leaflets. B: *esr2a* transcripts were not present in heart or liver. C: *esr2b* transcripts are in liver (arrow) but not in heart valves. Lateral views, anterior to the left. Scale bar = 50 μm.

Figure 4. Estrogen receptor subtype-specific activity causes tissue-specific response. 1-cell 5xERE:GFP embryos were injected with *esr2a* (A) or *esr2b* (B) antisense morpholino oligonucleotides (MO) to inhibit translation of ERβ1 or ERβ2 proteins. Embryos were exposed to 10 μM bisphenol A (BPA) at 2 days post fertilization and fluorescence was visualized a day later. Those injected with *esr2a* MO (A) exhibited fluorescence in the liver and heart valves, whereas those injected with *esr2b* MO (B) exhibited fluorescence in heart valves and not liver. C-E: The indicated ER subtype-specific agonists selectively activated the reporter in liver or heart valves. 3 day old 5xERE:GFP larvae were exposed to 10 μM BPA, 100 μM PPT or 1 μM DPN and fluorescence was visualized a day later. Arrows indicate liver, arrowheads indicate heart valves. All images are lateral views, anterior to the left, dorsal to the top. Scale bar = 100 μm.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Activity in heart and liver
Preferential activity in heart
No activity
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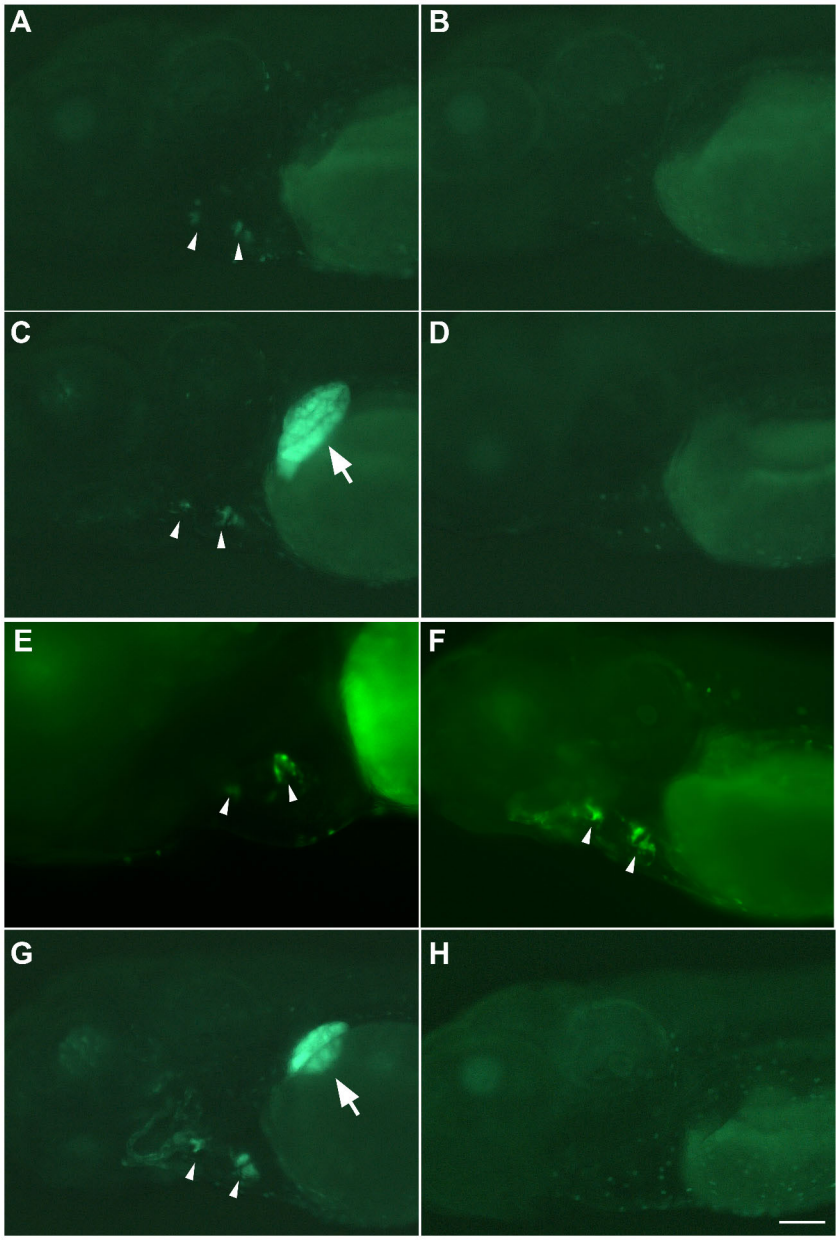
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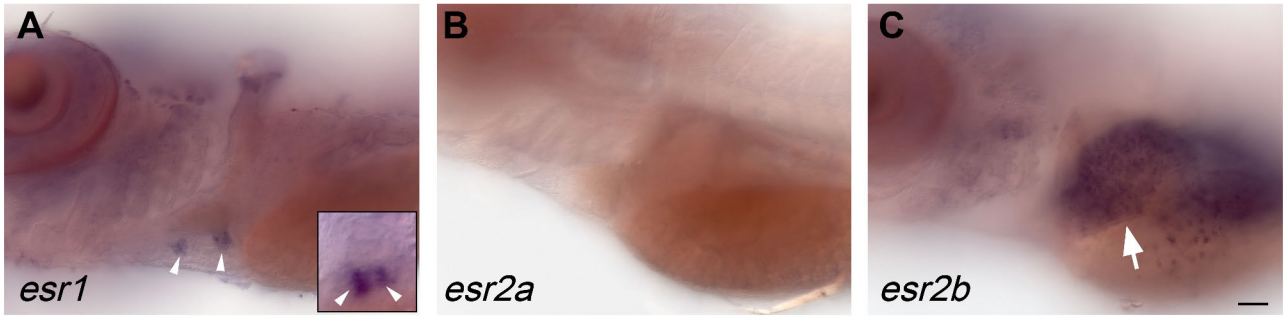
A
esr1
B
esr2a
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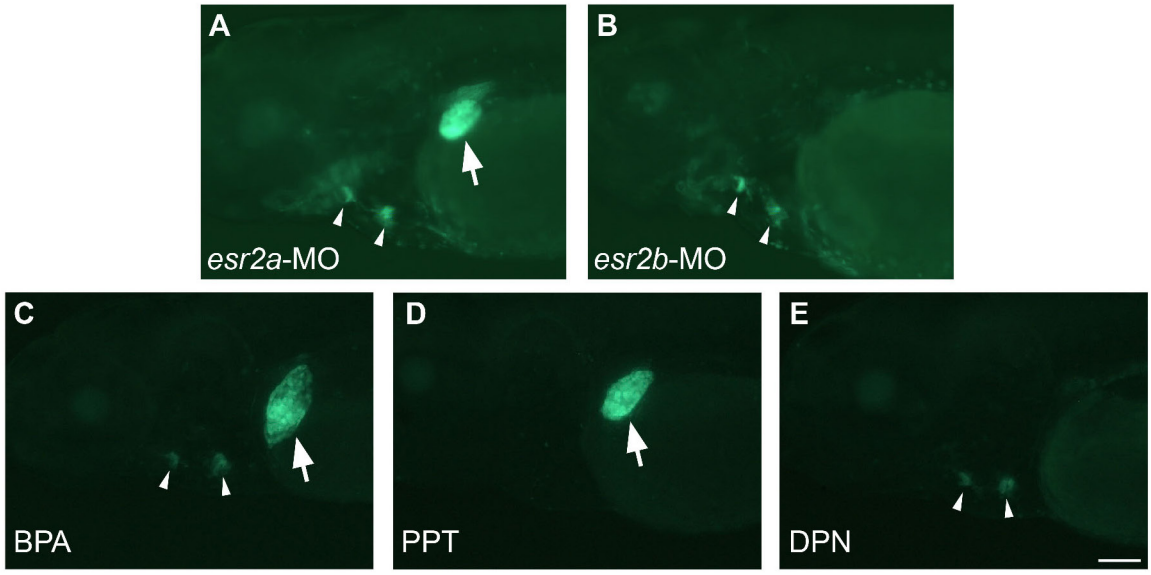
A
esr2a-MO
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